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DhhP, a Cyclic di-AMP Phosphodiesterase of *Borrelia burgdorferi*, Is Essential for Cell Growth and Virulence

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Cyclic di-AMP (c-di-AMP) is a recently discovered second messenger in bacteria. Most of work on c-di-AMP signaling has been done in Gram-positive bacteria, firmicutes, and actinobacteria, where c-di-AMP signaling pathways affect potassium transport, cell wall structure, and antibiotic resistance. Little is known about c-di-AMP signaling in other bacteria. *Borrelia burgdorferi*, the causative agent of Lyme disease, is a spirochete that has a Gram-negative dual membrane. In this study, we demonstrated that *B. burgdorferi* BB0619, a DHH-DHHA1 domain protein (herein designated DhhP), functions as c-di-AMP phosphodiesterase. Recombinant DhhP hydrolyzed c-di-AMP to pApA in a Mn^{2+}- or Mg^{2+}-dependent manner. In contrast to c-di-AMP phosphodiesterases reported thus far, DhhP appears to be essential for *B. burgdorferi* growth both *in vitro* and in the mammalian host. Inactivation of the chromosomal *dhhP* gene could be achieved only in the presence of a plasmid-encoded inducible *dhhP* gene. The conditional *dhhP* mutant had a dramatic increase in intracellular c-di-AMP level in comparison to the isogenic wild-type strain. Unlike what has been observed in Gram-positive bacteria, elevated cellular c-di-AMP in *B. burgdorferi* did not result in an increased resistance to β-lactamase antibiotics, suggesting that c-di-AMP's functions in spirochetes differ from those in Gram-positive bacteria. In addition, the *dhhP* mutant was defective in induction of the σ^S factor, RpoS, and the RpoS-dependent outer membrane virulence factor OspC, which uncovers an important role of c-di-AMP in *B. burgdorferi* virulence.

Nucleotide signaling molecules play a key role in regulating cellular activities throughout all kingdoms of life. Cyclic di-AMP (c-di-AMP) is a second messenger recently identified in *Bacteria* (for reviews, see references 1, 2, 3, 4, and 5). It was initially discovered in a structural study of the DisA protein (DNA integrity scanning protein) of *Bacillus subtilis* and *Thermotoga maritima*, where it was found bound to DisA (6). Cyclic di-AMP is synthesized from two molecules of ATP by diadenylate cyclase (DAC), which contains a conserved DisA_N domain (diadenylate cyclase, DUF147), and is hydrolyzed to pApA, or AMP, by phosphodiesterases (PDEs) containing the DHH (conserved Asp-His-His motif) and DHHA (DHH-associated domain) protein domains (5, 7). Genomic analyses revealed that proteins containing DisA_N and DHH-DHHA domains are encoded in the genomes of many *Bacteria* as well as some *Archaea*, suggesting that c-di-AMP signaling systems are widespread (1, 5).

Although the function of c-di-AMP remains poorly understood, it appears to be an essential molecule, since attempts to inactivate the DAC genes have failed in all bacterial species where they have been made (8–14). *B. subtilis* contains three DAC genes, but at least one of the three is needed for cell growth (15). On the other hand, the c-di-AMP-specific PDE genes proved to be dispensable, and the corresponding mutants have been obtained in several Gram-positive bacteria (*Firmicutes*), including *B. subtilis*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Listeria monocytogenes*, and *Lactococcus lactis* (7, 8, 16–18). Most of these PDEs belong to the GdpP protein family (GGDEF domain protein-containing Phosphodiesterase) (Fig. 1), as they contain N-terminal transmembrane helices linked to the highly degenerate GGDEF domain and PAS domain (Per-Arnt-Sim domain, known to be involved in binding to small-molecule metabolites) (19), followed by the DHH-DHHA1 domain module (20).

Studies on the c-di-AMP PDE-defective mutants indicate that increased intracellular c-di-AMP levels correlate with increased cell resistance to environmental stresses, such as acid stress, heat stress, UV irradiation, and antibiotics (8, 16, 17, 21, 22). Increased stress resistance may be due to an increased peptidoglycan cross-linking (8, 15). The *gdpP* mutant of *S. aureus* also rescued the severe growth defects of mutants lacking lipoteichoic acids (21). In addition, elevated intracellular levels of c-di-AMP also resulted in impaired cell size or cell division in *B. subtilis* (5), *S. aureus*, and *S. pneumoniae* (7, 15, 21). Deletion of the c-di-AMP-dependent transcriptional repressor DarR in *Mycobacterium smegmatis* also led to increased cell length (23). Recently identified RCK_C domain (regulator of conductance of K^+) was regarded as a c-di-AMP binding domain and a c-di-AMP binding protein (CabP) in *S. pneumoniae* interacting with a K^+ transporter has linked c-di-AMP with K^+ transport (24, 25).

In term of pathogenesis, c-di-AMP has been shown to function as PAMP (pathogen-associated molecular patterns), secreted through the multidrug resistance transporter (MTR) system of *L. monocytogenes* to the host cytosol, where it triggers STING (stimulator of interferon genes)-mediated activation of the interferon

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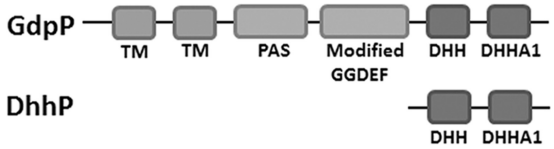

FIG 1 Diagram of the domain structure of the GdpP-family c-di-AMP PDEs and *B. burgdorferi* DhhP. TM, transmembrane domain.

(IFN) response. Inactivation of c-di-AMP PDE genes resulted in reduced infection in several pathogens, including *L. monocytogenes*, *S. pyogenes*, and *S. pneumoniae* (7, 8, 22, 26).

Virtually all the studies on c-di-AMP done thus far were conducted in Gram-positive bacteria, with the exception of one recent study showing that the Gram-negative bacterium *Chlamydia trachomatis* produces c-di-AMP (27). It was shown that c-di-AMP produced by *C. trachomatis* DAC is largely responsible for *Chlamydia*-induced STING-dependent type I IFN response in infected mammalian cells. However, the cellular functions of c-di-AMP in Gram-negative bacteria have not yet been examined.

Borrelia burgdorferi is a spirochetal pathogen and the causative agent of Lyme disease (28). Spirochetes are a phylogenetically distinct branch of *Bacteria*. Similar to other Gram-negative bacteria, spirochetes have a dual membrane structure with a thin layer of peptidoglycan located in the periplasmic space. The natural hosts of *B. burgdorferi* are ticks and mammals. *B. burgdorferi* undergoes massive transcriptome and proteome changes upon pathogen migration between these two drastically different hosts (29–31). Amazingly, *B. burgdorferi* accomplishes these dramatic processes with its streamlined genome and limited regulatory repertoire (32). For example, in contrast to many bacteria that have numerous c-di-GMP signaling systems (33–36), *B. burgdorferi* has only one c-di-GMP synthase (BB0419, diguanylate cyclase), and it plays an essential role in spirochetal survival in ticks as well as modulating *B. burgdorferi* motility and mammalian infection (37–43).

In this study, we began investigating the c-di-AMP signaling pathways of *B. burgdorferi*. We identified a gene for the DHH-DHHA1 domain protein BB0619, herein designated DhhP, in the *B. burgdorferi* genome and demonstrated that the protein functions as a c-di-AMP phosphodiesterase. Surprisingly, we found that, unlike what has been reported for Gram-positive bacteria, DhhP is essential for *B. burgdorferi* growth, and conditional inactivation of *dhhP* did not result in an increased resistance to β-lactam antibiotics, suggesting that c-di-AMP functions may differ in different phyla of bacteria. In addition, the conditional *dhhP* mutant is also defective in production of the major virulence factor OspC and in mammalian infection.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The low-passage, virulent *B. burgdorferi* strain 5A4NP1 (Table 1) (a gift from H. Kawabata and S. Norris, University of Texas Health Science Center at Houston) was derived from wild-type strain B31 by inserting a kanamycin resistance marker in the restriction modification gene *bbe02* on plasmid lp25 (44). *Borrelia* organisms were cultivated in Barbour-Stoenner-Kelly (BSK-II) medium supplemented with 6% normal rabbit serum (Pel Freez Biologicals, Rogers, AR) (45) at 37°C with 5% CO_2. Relevant antibiotics were added to the cultures with the following final concentrations: 250 μg/ml for kanamycin, 50 μg/ml for streptomycin, and 50 μg/ml for gentamicin. The constructed suicide vector (pMP001) was maintained in *Escherichia coli* strain DH5α. The antibiotics concentration used in *E. coli* were as follows: ampicillin, 100 μg/ml; chloramphenicol, 20 μg/ml; and gentamicin, 10 μg/ml.

Protein expression and purification. To obtain the recombination protein DhhP-6×His, the full-length *dhhP* (*bb0619*) gene was PCR amplified from *B. burgdorferi* strain B31 genomic DNA using the primer pair pLATE31-dhhP F/pLATE31-dhhP R (Table 2) and cloned into pLATE31 (Thermo Scientific aLICator LIC cloning and expression system) to generate pMP032. The DhhP-6×His fusion protein was expressed in BL21(DE3) and purified as previously described (46). Briefly, exponentially grown cells (A_{600}, 0.6) were induced for 5 h with 0.3 mM (final concentration) isopropyl-β-D-thiogalactopyranoside (IPTG) at 25°C. Cells were pelleted and resuspended in a buffer containing 300 mM NaCl, 50 mM NaH_2PO_4 (pH 7.4), 5% glycerol, and protease inhibitors (phenylmethylsulfonyl fluoride and P8465; Sigma, St. Louis, MO) at the concentrations specified by the manufacturer. Cells were subjected to disruption in a French pressure minicell (Spectronic Instruments), followed by brief sonication (Sonifier 250; Branson). Crude cell extracts were centrifuged at 35,000 × *g* for 25 min, and the supernatant was loaded onto Co^{2+} resin (Pierce) for affinity purification. After washing, proteins were eluted with 280 mM imidazole and desalted via Zeba Spin desalting columns (7-kDa cutoff; Pierce) that had been pre-equilibrated with buffer (300 mm NaCl, 0.5 mm EDTA, 10% glycerol, 50 mm NaH_2PO_4 [pH 7.4]). Protein purity was assessed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Protein concentration was measured using a Bradford protein assay kit (Bio-Rad).

Enzymatic assay. The phosphodiesterase assay conditions were essentially as described previously (17), with minor modifications. A reaction mixture (total volume, 0.6 ml) contained 0.5 μM DhhP and 50 μM c-di-AMP (Axxora) in the buffer 20 mM Tris (pH 8.0), 80 mM KCl, 10 mM metal cation (2.5 mM for Mn^{2+}). The reaction was carried out at 37°C. Aliquots were withdrawn and immediately boiled for 5 min, followed by centrifugation at 15,000 × *g* for 10 min. The supernatant was filtered through a 0.22-μm filter and analyzed by reversed-phase high-pressure liquid chromatography (HPLC) (Summit HPLC system; Dionex, Sunnyvale, CA) on a 15-cm by 4.6-mm Supelcosil LC-18-T column (Sigma) (47).

Measurements of intracellular c-di-AMP. *B. burgdorferi* organisms were grown in BSK-II medium to stationary phase, harvested in 4°C at 12,000 × *g* for 10 min, and immediately frozen in liquid nitrogen before being stored at −80°C. Nucleotides were extracted from frozen *B. burgdorferi* biomass by a mixture of 40% methanol–40% acetonitrile in 0.1 N formic acid as described earlier (48). The nucleotides were separated by HPLC and quantified by using liquid chromatography-tandem mass spectrometry (LC-MS/MS) on a Quattro Premier XE mass spectrometer (Waters Corporation) coupled with an Acquity Ultra Performance LC system (Waters Corporation) at the Michigan State Mass Spectrometry Core Facility. Cyclic di-AMP standards of known concentrations were

TABLE 1 *B. burgdorferi* strains used in this study

Strain	Description	Reference or source
5A4NP1	*B. burgdorferi* B31 with *bbe02* disrupted with a kanamycin-resistant marker	44
5A4NP1/pJJ01	5A4NP1 harboring an extra copy of the IPTG-inducible gene *dhhP*	This study
5A4NP1 *dhhP*/pJJ01	5A4NP1/pJJ01 with the endogenous gene *dhhP* disrupted by a gentamicin-resistant marker	This study

TABLE 2 Primers used in the study

Primer	Sequence (5′ → 3′)	Purpose
pLATE31-dhhP F	AGAAGGAGATATAACTATGAGAGATGTTATTAATTTTATT	Purify DhhP
pLATE31-dhhP R	GTGGTGGTGATGGTGATGGCCTAAATAAATATTATCCTTAATG	Purify DhhP
bb0619-UF	TTGCGAATTCTCCCAAGCTTAAAAGCTCTTCTTGT	Construct pMP001
bb0619-UR	TTCGGACGTCTCAAAATCAGGATCTTTGTGCCC	Construct pMP001
bb0619-DF	TCGAGACGTCAGATTTTGGAGGCGGTGGGCA	Construct pMP001
bb0619-DR	TAGCGGATCCAAGGGGGATTGGCGCAGCAC	Construct pMP001
pJJ01-F	CCATGGAGCTCTTTTGCGGAAGGTTTGCGAA	Construct pJJ01
pJJ01-R	GGCCCTCGAGTAAATAAATATTATCCTTAATGTATG	Construct pJJ01
dhhP ID-P1	ATGTTACGCAGCAGCAACGATGTTACGC	Verify the *dhhP* mutant
dhhP ID-P2	CTCCAGGATCCGTCGACTTTAGGTGGCGGTACTTGGGTCG	Verify the *dhhP* mutant
dhhP ID-P3	ATTTCGTGCTTTTTCTGCCAT	Verify the *dhhP* mutant
dhhP ID-P4	TGGAACAACACCCTTGGCAT	Verify the *dhhP* mutant
dhhP ID-P5	AGTCGGCCATTACGTGGAAA	Verify the *dhhP* mutant
dhhP ID-P6	ATCAAGAAGGGGGATTGGCG	Verify the *dhhP* mutant
dhhP ID-P7	TTCGGAGACGTAGCCACCTA	Verify the *dhhP* mutant
dhhP ID-P8	CAACAACCGCTTCTTGGTCG	Verify the *dhhP* mutant
dhhP ID-P9	AGAAGGAGATATAACTATGAGAGATGTTATTAATTTTATT	Verify the *dhhP* mutant
dhhP ID-P10	GTGGTGGTGATGGTGATGGCCTAAATAAATATTATCCTTAATG	Verify the *dhhP* mutant
qdhhP-F	GGGCTATATTGATCCTTTTGCACC	qRT-PCR
qdhhP-R	TGAAACCAGTCTTGCAACCA	qRT-PCR
qbosR-F	ACTGTTGCTTTTGATAGGCTTGG	qRT-PCR
qbosR-R	CGCATTGGAAAAAGTCGGCA	qRT-PCR

used for quantification. For intracellular c-di-AMP concentration measurements, the following simplifying assumptions were made: a *Borrelia* cell has a cylindrical shape 0.25 μm in diameter and 25 μm in length (49); therefore, an average cell volume was calculated to be 1.227×10^{-15} liter.

Construction of the conditional *dhhP* mutant. We first constructed a wild-type strain carrying a plasmid-borne copy of *dhhP* under the control of an IPTG-inducible promoter. First, the full-length *dhhP* gene was PCR amplified from 5A4NP1 genomic DNA using the primer pair pJJ01-F/pJJ01-R and cloned into an IPTG-inducible shuttle vector, pJJ275, a derivative of pJSB275 (50) with the addition of a hemagglutinin (HA) epitope tag (amino acid sequence, YPYDVPDYA) (51) at the C terminus of the inserted gene. The resulting plasmid was designed pJJ01. pJJ01 was then transformed into 5A4NP1 using the method described previously (52, 53). The final strain, 5A4NP1/pJJ01, was confirmed by immunoblotting with anti-HA antibody.

To inactivate the endogenous *dhhP* gene in 5A4NP1/pJJ01, a suicide vector, pMP001, was constructed for homologous recombination as following: the regions of DNA corresponding to 1.4 kb upstream and 1.4 kb downstream of *dhhP* were PCR amplified from 5A4NP1 genomic DNA with the primer pairs bb0619-UF/bb0619-UR and bb0619-DF/bb0619-DR (Table 2), respectively. The resulting PCR fragments were cloned into the vector pSC-A-amp/kan (Agilent Technologies) by T-A cloning and confirmed by restriction enzyme digestion and sequencing. The *aaC1* gentamicin-resistant marker driven by the *B. burgdorferi flaB* promoter (54) was digested out from pMH85R at the AatII sites (38) and was ligated between the upstream and downstream fragments of *dhhP* to generate the suicide vector pMP001. pMP001 was confirmed by both restriction enzyme digestion and sequencing and used for transformation. Cell lysates of positive *Borrelia* transformants were analyzed by PCR to confirm corrected marker insertion and inactivation of the chromosomal copy of *dhhP*. Plasmid profiles of the confirmed 5A4NP1 *dhhP*/pJJ01 clones were determined by multiple PCR analyses with 19 pairs of primers specific for each of the endogenous plasmids as reported by Bunikis et al. (55). One of the 5A4NP1 *dhhP*/pJJ01 clones that had plasmid profiles identical to those of the parental strain (5A4NP1) was chosen for further study.

Growth curve and cell morphology. *B. burgdorferi* strains, including the conditional *dhhP* mutant, were first grown in the presence of 1 mM IPTG. To avoid carryover of IPTG from the seed cultures used for inoculation, harvested cells were washed twice with PBS to remove residual IPTG. Then the IPTG-free cells were recultured in the fresh BSK-II medium with (5A4NP1 *dhhP*/pJJ01+IPTG) or without (5A4NP1 *dhhP*/pJJ01−IPTG) IPTG. Either a low (3×10^4 cells/ml) or high (3×10^5 cells/ml) dose of cells was used for inoculation (indicated in the figure legends). Cell numbers were numerated in every 12 or 24 h under dark-field microscopy. Cell morphology was visualized by dark-field microscopy, and the images were taken at a magnification of ×500. Ten different fields were selected for measuring the cell length of each strain, and 10 spirochetes in each field were randomly selected for measurement.

***In vitro* antimicrobial susceptibility test.** MICs of *B. burgdorferi* strains were determined by a standard broth microdilution method using 96-well tissue culture plates as previously described (56). Wild-type 5A4NP1, 5A4NP1 *dhhP*/pJJ01+IPTG, and 5A4NP1 *dhhP*/pJJ01−IPTG were incubated at 10^6 cells per ml in BSK-II medium containing a series of 2-fold dilutions of ampicillin, aztreonam, cefuroxime, cefixime, vancomycin, and oxacillin (a total volume of 220 μl per well). The culture plates were sealed with Parafilm and were incubated for 72 h at 37°C. The lowest concentration of antibiotics that showed inhibition (lack of a color change of the BSK II medium) was interpreted as the MIC.

Quantitative RT-PCR (qRT-PCR) and qPCR. RNA samples were extracted from *B. burgdorferi* cultures using the RNeasy minikit (Qiagen, Valencia, CA) according to the manufacturer's protocols. Three independent culture samples were used for each strain. Digestion of contaminating genomic DNA in RNA samples was performed using RNase-free DNase I (Promega), and removal of DNA was confirmed by PCR amplification for the *B. burgdorferi flaB* gene. cDNA was synthesized using the SuperScript III reverse transcriptase with random primers (Invitrogen, Carlsbad, CA). To quantify the transcript levels of genes of interest, an absolute quantitation method was used by creating a standard curve in qPCR assay by following the manufacturer's protocol (Stratagene, La Jolla, CA). Briefly, the PCR product of *recA* gene served as a standard template. A series of 10-fold dilutions (10^2 to 10^8 copies/ml) of the standard template was prepared, and qPCR was performed to generate a standard curve by plotting the initial template quantity against the cycle threshold (C_T) values for the standards. The quantity of the targeted genes in cDNA samples was calculated using their C_T values and the standard

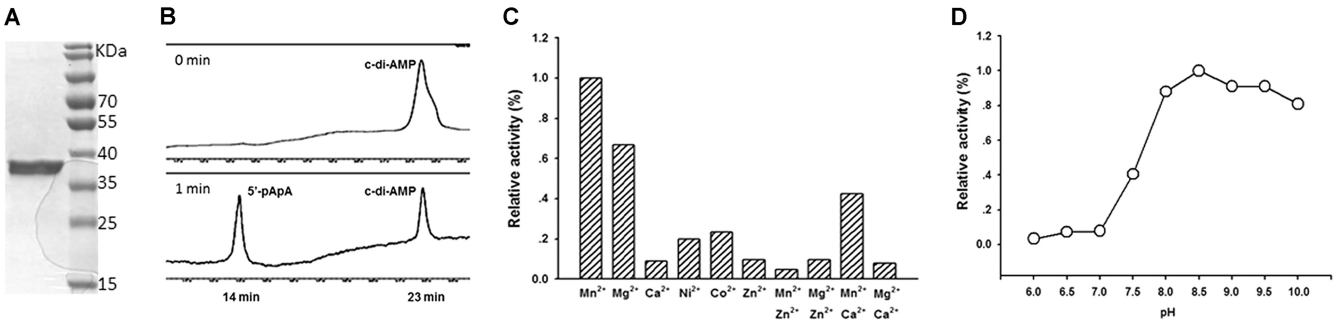

FIG 2 Phosphodiesterase activity of *B. burgdorferi* DhhP *in vitro*. (A) DhhP-6×His protein following affinity purification. (B) Cyclic di-AMP hydrolysis by *B. burgdorferi* DhhP monitored by HPLC. The reaction mixture contained 0.5 μM DhhP and 50 μM c-di-AMP in the buffer (20 mM Tris, 80 mM KCl, 2.5 mM Mn^{2+} [pH 8.0]) and was incubated at 37°C. Nucleotides were separated and analyzed using reversed-phase HPLC. Shown are nucleotide profiles before (0 min) and after (1 min incubation) addition of DhhP. (C) Metal cation dependence of c-di-AMP hydrolysis activity of DhhP. (D) pH dependence of c-di-AMP hydrolysis activity of DhhP.

curve. Samples were performed in triplicate using the ABI 7000 sequence detection system and green PCR master mix (ABI, Pleasanton, CA). Levels of the target gene transcript were reported per 100 copies of *recA* transcripts.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Spirochetes were harvested by centrifugation at 8,000 × *g* for 10 min and washed three times with PBS (pH 7.4) at 4°C. Pellets were suspended in SDS buffer containing 50 mM Tris-HCl (pH 8.0), 0.3% sodium dodecyl sulfate (SDS), and 10 mM dithiothreitol (DTT). Cell lysates (5×10^7 cells per lane) were separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes (GE-Healthcare, Milwaukee, WI). Membranes were blotted with monoclonal antibodies against FlaB, OspC, RpoS, and BosR (57–59) with 1:500, 1:1,000, 1:50, and 1:500 dilutions, respectively, and then with goat anti-mouse IgG horseradish peroxidase (HRP)-conjugated secondary antibody (1:1,000; Santa Cruz Biotechnology). Detection of horseradish peroxidase activity was determined by the enhanced chemiluminescence method using luminol and peroxide as the substrate (Thermo Pierce ECL Western blotting substrate) and subsequently by exposure to X-ray film.

Dialysis membrane chamber (DMC) implantation and mouse infection. For cultivation of *B. burgdorferi* in the DMC mammalian host model, 5 ml of spirochetes (10^3 organisms per ml) in BSK-II medium were inoculated into dialysis tubing (Fisherbrand regenerated cellulose) and implanted into the peritoneal cavities of female Sprague-Dawley rats (two tubes per rat) (60, 61). For the rat implanted with 5A4NP1 *dhhP*/pJJ01+IPTG, 10 mM IPTG was added to the drinking water (62). Dialysis tubing was harvested 14 days after implantation, and the growth of spirochetes were determined by examination of the cultures under the dark-field microscope.

For mouse infection, 3- or 4-week-old C3H/HeN mice (Harlan, Indianapolis, IN) were subcutaneously inoculated with 1×10^5 spirochetes. For mice infected with 5A4NP1 *dhhP*/pJJ01+IPTG, 10 mM IPTG was added to the drinking water and given via intraperitoneal (IP) injection (100 μl of 10 mM IPTG) every 2 days (62). Ear punch biopsy specimens were collected 14 days after infection and cultivated in 2 ml of BSK-II medium (Sigma-Aldrich, St. Louis, MO) containing a mixture of phosphomycin (2 mg/ml), rifampin (5 mg/ml), and amphotericin B (250 mg/ml) (Sigma-Aldrich). All cultures were maintained at 37°C and examined for the presence of spirochetes by dark-field microscopy beginning 5 days after inoculation. A single growth-positive culture was used as the criterion to determine positive mouse infection.

RESULTS

***B. burgdorferi* DhhP (BB0619) is a c-di-AMP phosphodiesterase.** The c-di-AMP-specific phosphodiesterases (PDE) studied thus far are from Gram-positive bacteria and belong to the GdpP family (5, 7, 17, 26). GdpP often contains a DHH-DHHA1 domain together with several regulatory domains (Fig. 1). The C-terminal DHH-DHHA1 module is responsible for c-di-AMP cleavage (17). A BLAST search revealed that the *B. burgdorferi* B31 genome encodes a single DHH-DHHA1-containing protein, BB0619, that lacks other regulatory domains (Fig. 1). We designated it DhhP (DHH-DHHA1 domain protein).

To investigate whether DhhP has c-di-AMP phosphodiesterase activity, a C-terminally 6×His-tagged recombinant DhhP was expressed and purified (Fig. 2A). DhhP was then incubated with c-di-AMP in reaction buffers containing various metal cations. The result showed that DhhP hydrolyzed c-di-AMP to 5′-pApA, with the maximum activity being observed with Mn^{2+} as a cofactor, and the optimal pH of 8.5 (Fig. 2B to D), similar to the observations made for other c-di-AMP PDEs (5, 17). Mg^{2+} could also function as an effective metal cofactor, but Zn^{2+} and Ca^{2+} were inhibitory. Thus, we conclude that *B. burgdorferi* DhhP has a c-di-AMP phosphodiesterase activity.

DhhP is essential for cell growth *in vitro*. To investigate the function of DhhP in *B. burgdorferi*, we initially attempted to inactivate *dhhP* in wild-type *B. burgdorferi*. However, multiple attempts were unsuccessful. We reasoned that *dhhP* may be essential for *B. burgdorferi*, despite the fact that other c-di-AMP PDE genes have been readily inactivated in other bacteria (8, 16, 17, 21, 22). To test this hypothesis, we performed a conditional knockout of *dhhP* by first transforming the shuttle vector (pJJ01) carrying an IPTG-inducible *dhhP* gene into wild-type *B. burgdorferi* strain 5A4NP1, resulting in strain 5A4NP1/pJJ01 (Fig. 3A). Then the suicide vector used for inactivation of *dhhP* was transformed into 5A4NP1/pJJ01 in the presence or absence of IPTG. Ninety-three positive colonies were obtained with one independent transformation when IPTG was included in the medium, while no positive colonies were obtained with 5A4NP1 or 5A4NP1/pJJ01−IPTG. PCR analyses confirmed that these positive clones were conditional *dhhP* mutants that contain a disrupted endogenous *dhhP* gene and an inducible *dhhP* on the shuttle vector (Fig. 3B). The endogenous plasmid profiles of the conditional *dhhP* mutant were determined using the method developed by Bunikis et al. (55). One of the *dhhP* mutant clones that had plasmid profiles identical to those of the parental strain, 5A4NP1, was chosen for further study (Fig. 3C).

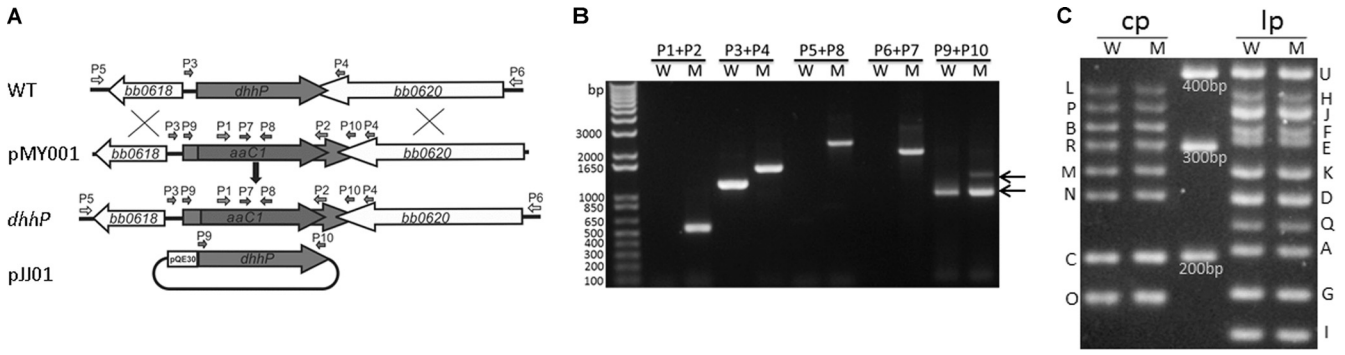

FIG 3 Construction of the conditional *dhhP* mutant. (A) Strategy for constructing the *dhhP* mutant. Arrows indicate the positions of primers (labeled above the arrows) for PCR analyses. (B) PCR analysis of the wild-type (W) and mutant (M) strains. The specific primer pairs used in PCR are indicated at the top. The arrows indicate the disrupted endogenous *dhhP* located in the chromosome (upper arrow) and an inducible copy of *dhhP* located on the shuttle vector (lower arrow). (C) Endogenous plasmid profiles of the wild-type (W) and mutant (M) strains obtained by PCR analysis using the method published by Bunikis et al. (55). cp, circular plasmid; lp, linear plasmid. Letters on each side indicate the bands corresponding to each endogenous plasmid defined previously for *B. burgdorferi* strain B31 (32).

To confirm that *dhhP* is essential for *B. burgdorferi* growth, wild-type and the conditional *dhhP* mutant strains grown in the presence of IPTG were first harvested and washed to remove IPTG. The IPTG-free cells were then inoculated into fresh BSK-II medium supplemented with various concentrations of IPTG. The result showed that the growth of the conditional *dhhP* mutant was IPTG dependent (Fig. 4A). When the initial inoculated culture concentration was high (3×10^5 spirochetes per ml) and the IPTG concentration was less than 10 μM, the *dhhP* mutant replicated 5 or 6 times and then stopped (reaching about 1×10^7 spirochetes per ml) (Fig. 4A). Supplementing the medium with >10 μM IPTG restored the growth of the *dhhP* mutant to the level of wild-type spirochetes. To investigate if the *dhhP* mutant could reach a density of 10^7 cells/ml, we inoculated the culture at a lower cell density (3×10^4 cells/ml). As shown in Fig. 4B, when IPTG was absent, the *dhhP* mutant replicated a few times and stopped, and it was not able to grow to 10^7 cells/ml.

We reasoned that the initial small number of replications observed for the conditional *dhhP* mutant observed in Fig. 4A was likely due to the presence of DhhP or IPTG in the initial cells used for inoculation. After cells had replicated several times, DhhP was depleted or degraded, and the cells then stopped replicating. To test this possibility, we used stationary-phase cells (Fig. 4A, arrow) for inoculation into fresh IPTG-free BSK-II medium at a higher dose (3×10^5 per ml). As shown in Fig. 4C, the *dhhP* mutant was no longer able to replicate even for the first few times. Note that these cells remained viable under dark-field microscope (data not shown), and they replicated normally in the medium supplemented with 1 mM IPTG (Fig. 4C). Taking these data together, we conclude that DhhP is indispensable for the growth of *B. burgdorferi*.

Deletion of *dhhP* results in an elevated cellular c-di-AMP level. We first confirmed that the conditional *dhhP* mutant no longer expressed *dhhP*. mRNA was extracted from wild-type cells or 5A4NP1 *dhhP*/pJJ01 cells grown with and without IPTG at the stationary phase (Fig. 4A, arrow). As shown in Fig. 5A, in the absence of IPTG, the conditional mutant had no detectable level of *dhhP* transcript by RT-PCR analysis. We then subjected the cells harvested under the same conditions to measurement of the cellular concentrations of c-di-AMP. Given that DhhP was predicted to be the only DHH-DHHA-encoding protein in the *B. burgdorferi* genome, inactivation of *dhhP* was expected to result in an increased level of c-di-AMP. Indeed, the level of c-di-AMP increased more than 40-fold in the conditional *dhhP* mutant without IPTG over that in the wild-type *B. burgdorferi* strain (Fig. 5B). This result provided *in vivo* evidence that DhhP acts as a c-di-AMP PDE in *B. burgdorferi*.

DhhP is essential for *B. burgdorferi* growth *in vivo*. The data

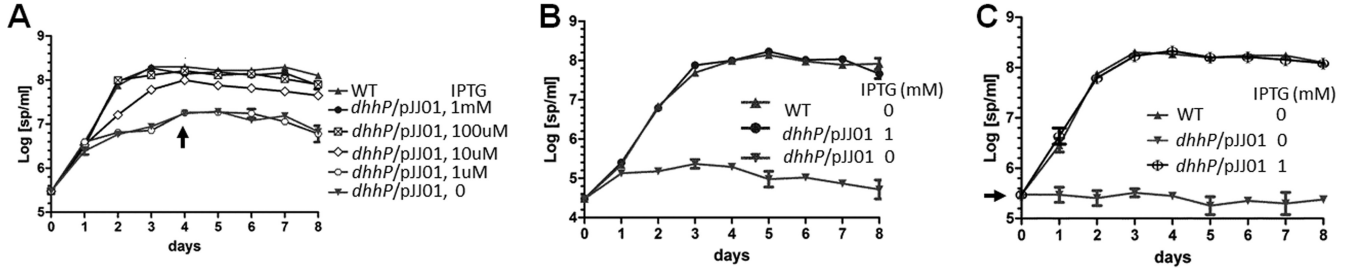

FIG 4 The *dhhP* mutant has impaired growth. (A) Wild-type strain and the conditional mutant 5A4NP1 *dhhP*/pJJ01 were cultured in BSK-II medium with 1 mM IPTG, harvested at mid-log phase, and then washed twice with fresh BSK-II medium to remove residual IPTG. The washed cells were then recultured in BSK medium with various concentrations of IPTG. The initial cell density was 3×10^5 cells/ml. Spirochetes were enumerated under a dark-field microscope. Each data point is the average of data from three independent cultures. The arrow indicates the time point taken for the next experiment (C), when cells were at stationary phase. (B) Same as panel A except that the cultures were inoculated at a lower cell density (3×10^4 cells/ml). (C) Reinoculation of the stationary-phase cells from the culture used for panel A into fresh BSK-II medium.

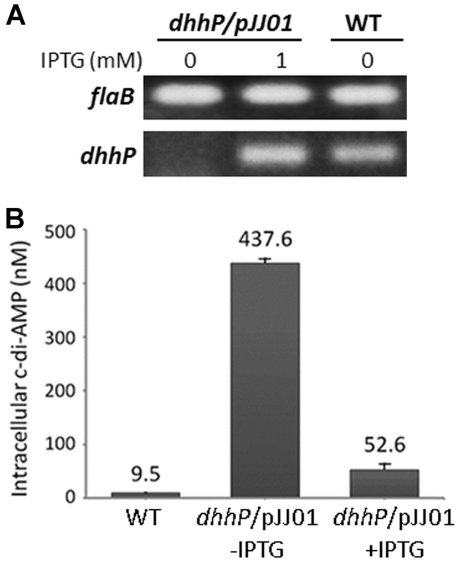

FIG 5 Inactivation of *dhhP* results in an increased intracellular c-di-AMP level. (A) Confirmation of inactivation of *dhhP* by RT-PCR analyses. Wild-type spirochetes or the conditional *dhhP* mutant grown in the absence or presence (1 mM) of IPTG were taken from cultures at day 4 (Fig. 4A). (B) Intracellular c-di-AMP concentrations. The IPTG concentration was 1 mM. Nucleotides were extracted from frozen *B. burgdorferi* organisms, separated by HPLC, and quantified by using tandem mass spectrometry as described in Materials and Methods.

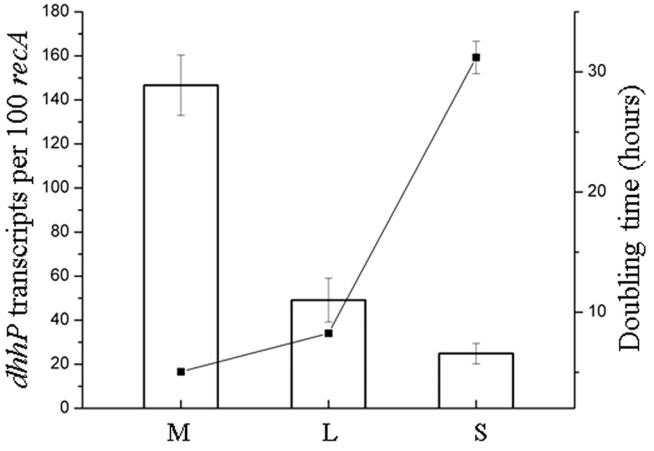

FIG 6 Growth phase-dependent *dhhP* expression. Wild-type strain 5A4NP1 was grown in standard BSK-II medium at 37°C and harvested at different stages of growth. M, mid-logarithmic phase; L, late logarithmic phase; S, stationary phase. Bars represent *dhhP* mRNA levels, and black dots represent the culture doubling times. RNA was extracted and subjected to RT-PCR analysis. The data are from one representative experiment with three independent cultures. Each data point is the average from three independent cultures.

above showed that the *dhhP* mutant has an impaired growth phenotype under the *in vitro* cultivation conditions. To determine whether DhhP is required for *B. burgdorferi* replication in the mammalian host environment, the wild type and the 5A4NP1 *dhhP*/pJJ01 strains with and without IPTG were grown in dialysis membrane chambers (DMCs) implanted in rat peritoneal cavities, which were developed by Akins et al. (60). For the IPTG group, 10 mM IPTG was also provided in rat drinking water (62). Fourteen days after cultivation, DMCs were harvested and the cultures were examined under a dark-field microscope. As shown in Table 3, the wild type and the conditional *dhhP* mutant spirochetes supplemented with IPTG were readily detectable in DMC cultures, whereas the *dhhP* mutant without IPTG was not detected in DMCs.

We further performed mouse infection experiments for the conditional *dhhP* mutants. The wild type and the 5A4NP1 *dhhP*/pJJ01 strains were needle inoculated into groups of mice (1×10^5 spirochetes per mouse). One group of the mice inoculated with 5A4NP1 *dhhP*/pJJ01 was provided with 100 μl 10 mM IPTG by IP every 2 days. Fourteen days after inoculation, ear punch biopsy specimens were collected from mice for cultivation of spirochetal growth. All mice inoculated with either wild-type *B. burgdorferi* or 5A4NP1 *dhhP*/pJJ01 supplied with IPTG were infected. No mice inoculated with 5A4NP1 *dhhP*/pJJ01 without IPTG were infected. Taken together, these results indicate that DhhP is essential for *B. burgdorferi* replication both *in vitro* and *in vivo*.

TABLE 3 *dhhP* is required for *Borrelia* growth *in vivo*

Strain	Growth in DMC	No. of infected mice/total
5A4NP1	+	5/5
5A4NP1 *dhhP*+IPTG	+	4/4
5A4NP1 *dhhP*−IPTG	−	0/6

Growth phase-dependent expression of *dhhP*. Given that DhhP plays an essential role in *B. burgdorferi* growth, we investigated whether *dhhP* expression correlates with the growth rate of *B. burgdorferi*. Wild-type *B. burgdorferi* 5A4NP1 spirochetes grown in standard BSK-II medium at 37°C were harvested at different growth phases and subjected to qRT-PCR analyses. As shown in Fig. 6, the *dhhP* transcript level correlated with the growth rate. Growth rate-dependent expression of *dhhP* is consistent with its essential role in *B. burgdorferi* growth.

The *dhhP* mutant has elongated cells. Previous reports indicated that alterations in cellular c-di-AMP were associated with changes in cell morphology. We observed that the conditional *dhhP* mutant spirochetes grown in the absence of IPTG had exhibited five times longer cells than the wild type or the 5A4NP1 *dhhP*/pJJ01 spirochetes grown in the presence of IPTG (Fig. 7), suggesting that DhhP plays an important role in cell division.

The *dhhP* mutant does not have increased resistance to β-lactamase antibiotics. Increased intracellular c-di-AMP concentration has been shown to affect cell wall/peptidylglycan structure and lead to increased resistance to β-lactam antibiotics in several Gram-positive bacteria, including *B. subtilis*, *S. aureus*, *S. pyogenes*, and *L. monocytogenes*. To examine whether deletion of *dhhP* affects *B. burgdorferi* susceptibility to cell wall-targeting antimicrobials, MICs of the conditional *dhhP* mutant were measured using the broth microdilution method in 96-well tissue culture plates (56). The wild type and 5A4NP1 *dhhP*/pJJ01 with or without IPTG were incubated with a series of dilutions of ampicillin, aztreonam, cefuroxime, cefixime, vancomycin, and oxacillin at 37°C. Note that we inoculated cells at a high dose (10^6 cells per ml) so that the growth of the conditional *dhhP* mutant could be observed. MICs were calculated 72 h after incubation.

Unlike findings made with Gram-positive bacteria, in which c-di-AMP *pde* inactivation resulted in an enhanced antibiotic re-

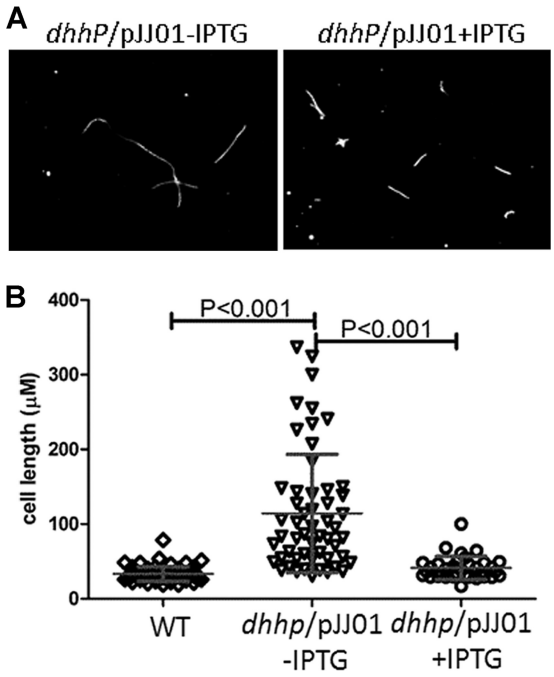

FIG 7 The *dhhP* mutant is defective in cell division. (A) Morphology of 5A4NP1 *dhhP*/pJJ01 grown with IPTG or 5A4NP1 *dhhP*/pJJ01 grown without IPTG examined under a dark-field microscope. Images were taken at a magnification of ×500. (B) Quantitation of cell length. A total of 100 randomly selected cells from each strain were measured.

sistance, the conditional *dhhP* mutant of *B. burgdorferi* did not exhibit a dramatic increase in resistance (and showed slightly reduced resistance [2×] to some β-lactam antibiotics) (Table 4). These results suggest that c-di-AMP may function differently in spirochetes than in Gram-positive bacteria.

The *dhhP* mutant is defective in producing the major virulence factor OspC. To examine the impact of deletion of *dhhP* on the overall protein profile of *B. burgdorferi*, whole-cell lysates from various strains were harvested and subjected to SDS-PAGE analysis (Fig. 8A). In comparison to the *dhhP* mutant grown in the presence of IPTG, the same mutant in the absence of IPTG had much lower OspC levels. Since *ospC* expression is controlled by RpoS, we further examined the levels of RpoS by immunoblotting. As expected, RpoS was induced in the conditional *dhhP* mutant with IPTG, with an increase of cell density (Fig. 8A). However, RpoS was not detectable in the *dhhP* mutant in the absence of IPTG.

It is well established that expression of *rpoS* is governed by two transcriptional factors, Rrp2 and BosR (63–65). Regulation of Rrp2 occurs at the level of phosphorylation. Since a method to assess the level of Rrp2 phosphorylation within the cell is lacking, a potential impact of DhhP on Rrp2 phosphorylation could not be examined. Therefore, we focused on regulation of BosR (43, 57, 66). Similar to what was seen with the levels of RpoS, the *dhhP* mutant failed to induce BosR production in the absence of IPTG, indicating that the loss of OspC and RpoS in the *dhhP* mutant was due to a defect in BosR production (Fig. 8A). As expected, the *dhhP* mutant had no *dhhP* mRNA and had a significantly reduced *rpoS* mRNA level (Fig. 8B and C). However, the *bosR* mRNA level was only moderately reduced, with no statistical significance, in the *dhhP* mutant (Fig. 8D), suggesting that deletion of *dhhP* affects BosR mainly at the posttranscriptional level, a phenomenon that has been observed under several other conditions (39, 57, 66). Thus, DhhP appears to be required for BosR accumulation, which in turn controls production of RpoS and RpoS-dependent virulence factors, including OspC.

DISCUSSION

Virtually all reported studies on the c-di-AMP signaling thus far have focused on Gram-positive bacteria, with one exception showing that the Gram-negative pathogen *C. trachomatis* is also capable of producing c-di-AMP (27). In this study, we showed that c-di-AMP signaling is also present in the phylum *Spirochaetae*. We demonstrated, *in vitro* and *in vivo*, that the DHH-DHHA1 protein in *B. burgdorferi*, DhhP, functions as a c-di-AMP PDE. Unlike what has been observed in Gram-positive bacteria, DhhP is required for cell growth of *B. burgdorferi*.

The requirement of DhhP for growth of *B. burgdorferi* was surprising, given that none of the c-di-AMP PDEs has been shown to be essential for cell growth, but rather, it has been demonstrated that diadenylate cyclase is indispensable for cell survival in all Gram-positive bacteria examined. Furthermore, the reported PDE-defective mutants (containing increased intracellular c-di-AMP levels) have elevated resistance to peptidoglycan-targeted antibiotics in *S. aureus* (21), *B. subtilis* (15), *S. pyogenes* (26), and *L. monocytogenes* (8). However, deletion of *dhhP* of *B. burgdorferi* did not result in an increased resistance to cell wall-targeting antibiotics (Table 4). Since increased antibiotic resistance in c-di-AMP PDE mutants has been linked to an increased amount of cross-linked peptidoglycan in *S. aureus* (21), we also examined the *dhhP* mutant of *B. burgdorferi* using cryo-electron microscopy in collaboration with Jun Liu at the University of Texas at Houston. We did not observe any significant change in the thickness of the peptidoglycan layer (data not shown). Another phenotype of the *dhhP* mutant that is different from the phenotypes of other PDE mutants in Gram-positive bacteria concerned differences in cell shape. The *dhhP* mutant of *B. burgdorferi* had an increased cell length, whereas inactivation of *gdpP* resulted in a reduced cell size of *S. aureus* (21). In *B. subtilis*, curled nonseparated cell filaments were observed upon cellular accumulation of c-di-AMP (67). All these findings suggest that c-di-AMP may function differently in modulating cell wall homeostasis of *B. burgdorferi*. As spirochetes

TABLE 4 MIC of *B. burgdorferi* strains for various antibiotics

Strain	MIC (μg/ml) of:					
	Ampicillin	Aztreonam	Cefuroxime	Cefixime	Vancomycin	Oxacillin
5A4NP1	2.5	25	0.156	0.625	0.625	1.25
5A4NP1 *dhhP*/pJJ01+IPTG	2.5	25	0.156	0.625	0.625	1.25
5A4NP1 *dhhP*/pJJ01−IPTG	1.25	25	0.156	0.625	0.312	0.625

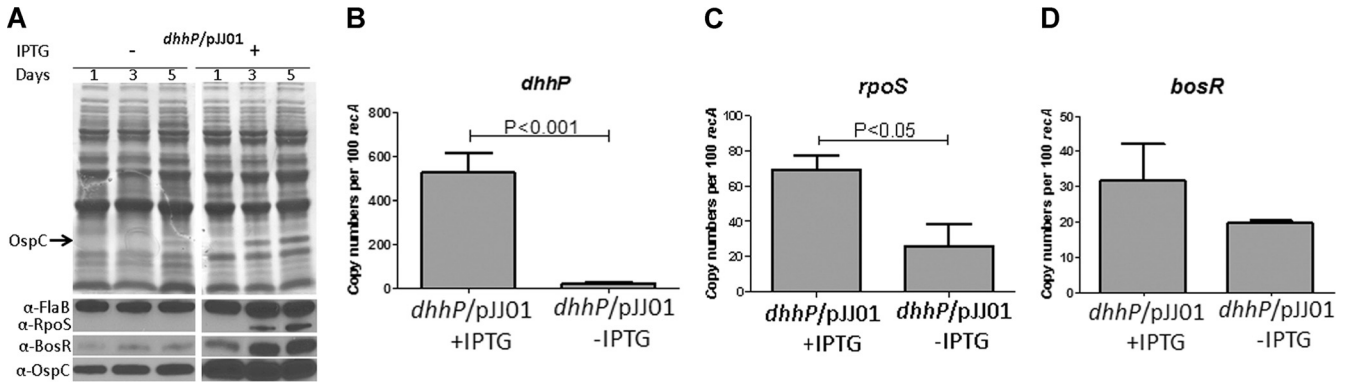

FIG 8 The *dhhP* mutant is defective in the production of the major virulence factor OspC. (A) The conditional *dhhP* mutant was inoculated at a higher cell density (3×10^5 cells/ml) in BSK-II medium with or without IPTG at 37°C. Cells were harvested at different days (labeled at the top) and subjected to SDS-PAGE and immunoblotting analyses. FlaB served as a loading control. The bands corresponding to OspC are indicated by an arrow. (B to D) qRT-PCR assessment of expression of *dhhP* (B), *rpoS* (C), and *bosR* (D). RNA was extracted from cells harvested at day 3 and subjected to qRT-PCR analyses. Levels of expression of each gene were normalized with *recA*.

have a cell wall structure similar to that of Gram-negative bacteria (with dual membranes), it will be interesting to investigate the roles of c-di-AMP phosphodiesterases in proteobacteria that appear to possess c-di-AMP signaling pathways (5).

Most of the c-di-AMP PDEs reported thus far have been grouped into the GdpP family, including *B. subtilis* YybT (17), *S. aureus* GdpP, *S. pyogenes* GdpP (26), and *S. pneumoniae* Pde1 (7). Besides DHH-DHHA1 domains responsible for c-di-AMP hydrolysis, proteins in the GdpP family contain an additional degenerate GGDEF domain and PAS domain (5). An intact GGDEF domain is responsible for diguanylate cyclase activity involved in synthesis of c-di-GMP (34, 68, 69). The GGDEF domain within GdpP, however, has no such activity but is capable of hydrolyzing ATP (17) and may regulate the activity of the DHH-DHHA1 domains (21). The PAS domain in GdpP has been shown to bind to heme and plays a regulatory role in c-di-AMP PDE's activity (70). These proteins also have transmembrane domains and are associated with the cell membrane (5). In this regard, *B. burgdorferi* DhhP has a single DHH-DHHA1 domain module and lacks transmembrane and regulatory domains. This domain structure suggests that DhhP is a cytoplasmic protein and its activity may be regulated at the level of gene expression, not at the level of its enzymatic activity. In this study, we showed that *dhhP* expression is growth phase dependent, being highest during the exponential phase (Fig. 6).

Given that the *dhhP* mutant has an impaired growth phenotype under the *in vitro* cultivation conditions, it was not surprising that the same mutant was not able to replicate in the dialysis membrane implanted in the rat peritoneal cavity (the DMC conditions) or when directly inoculated into mice. These results suggest that the requirement for DhhP does not depend on the environmental niches where *B. burgdorferi* resides, and elevated levels of c-di-AMP likely inhibit one or more cellular processes that are essential for *B. burgdorferi* growth. One of the cellular processes affected by c-di-AMP may be cell division, as the conditional c-di-AMP mutant showed increased cell length (Fig. 7). The *dhhP* mutant was also unable to produce RpoS and RpoS-dependent virulence factors, such as OspC. This defect could be simply due to its growth defect, as induction of RpoS and OspC is growth rate dependent (71).

This study focused on c-di-AMP PDE of *B. burgdorferi*. Inactivation of *dhhP* resulted in accumulation of cellular c-di-AMP, indicating that *B. burgdorferi* is capable of producing the second messenger c-di-AMP. The enzyme diadenylate cyclase (DAC) for c-di-AMP synthesis contains a conserved DisA_N domain (21). In this regard, we have found that the hypothetical protein BB0008 in the *B. burgdorferi* genome encodes a putative DisA_N domain (E value = 5.45e-34), which makes BB0008 a strong candidate as a DAC of *B. burgdorferi*. Currently, no information is available about BB0008; its expression is not regulated by temperature, DMC conditions, or the two-component system Hk1-Rrp1 or Hk2-Rrp2 based on the published microarray data (37, 38, 72–79). We are currently in the process of investigating the role of BB0008 in the c-di-AMP signaling of *B. burgdorferi*.

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